Journal of Asian Scientific Research
ISSN(e): 2223-1331
ISSN(p): 2226-5724
DOI: 10.55493/5003.v12i1.4449
Vol. 12, No. 1, 12-27.
© 2022 AESS Publications. All Rights Reserved.
URL: www.aessweb.com

CALLUS INDUCTION OF FENUGREEK *TRIGONELLA FOENUM-GRAECUM* VIA AUXIN COMBINED WITH CYTOKININS HORMONES, AND ASSESSMENT OF TOXICITY VIA BRINE SHRIMP ASSAY

Hasan M. Agha[1]
Khairul Adzfa Radzun[2+]
Norrizah Jafaar Sidik[3]
Ali H. Jawad[4]

[1,2,3,4]*Faculty of Applied Sciences, University of Technology Mara, Malaysia.*
[1]*Email: hasanagha586@gmail.com Tel: +601111666409*
[2]*Email: khairuladzfa@uitm.edu.my Tel: +601112275773*
[3]*Email: norri536@uitm.edu.my Tel: +60132626737*
[4]*Email: ahjm72@gmail.com Tel: +60164045785*

(+ Corresponding author)

Article History
Received: 11 January 2022
Revised: 2 March 2021
Accepted: 16 March 2021
Published: 25 March 2022

Keywords
Fenugreek
Trigonella foenum-graecum
Callus induction
Auxin
Cytokinins
Toxicity
Brine shrimp assay.

ABSTRACT

Trigonella foenum-graecum (TFG) is a significant leguminous plant with diverse pharmacological effects. However, the resistant character of this plant accounts for significant difficulties in vitro multiplication, justifying the necessity to try new techniques for in vitro propagation of this plant. Hence, this study reports the effects of BAP, NAA, and 2,4-D on in vitro callus formation of seeds. The toxicity properties of seeds, plant, and callus aqueous extracts of TFG were measured by brine shrimp assay (BSA). Callus index, frequency of callus, callus weight, and morphology of callus were recorded after 30 days of culture. No callus formation was observed in the absence of plant growth regulators. The maximum callus formation observed in the MS media containing 1.0 mg/l 2,4-D, the highest mean of the callus index (52±9.5) with 100% frequency and callus yield (0.52±0.08 g) in 30 days of culture. The highest mean of callus index (37±0 4.05) for combination hormones with 100% callusing and yield (0.37±0.02 g) in 30 days of culture by 1.0 mg/l BAA with 0.5 mg/l NAA. Seeds extract of TFG showed the highest toxicity (954.99 µg/ml), aqueous plant extract (1237.98 µg/ml), and aqueous callus extract (1801 µg/ml) from BSA. Comparing individual hormones, the highest amount of callus in TFG can be yielded 2,4-D hormone alone, and a combination of BAP and NAA can yield 100% callus.

Contribution/ Originality: This study contributes to the existing literature in plant tissue culture techniques and determines various concentrations of hormones, where this study has been done by using two groups of hormones auxin and cytokinins. The seeds of fenugreek were cultured in media supplemented with BAP, NAA, and 2,4-D hormones.

1. INTRODUCTION

Plant tissue culture is the in vitro growing of plant cells or tissues in an aseptic and controlled environment, in liquid or semisolid well-specified nutritional media, to synthesise primary and secondary metabolites or regenerate plants [1, 2]. This approach provides alternate answers to problems that arise due to the current pace of extinction and devastation of flora and ecosystems. The entire procedure necessitates a well-equipped culture laboratory and nutritional media [3, 4].

Plant callus is a collection of undifferentiated cells generated from plant tissue (explants) in biological research and biotechnology. Callus cells over a plant wound in plant biology [5]. Callus development from original explants

© 2022 AESS Publications. All Rights Reserved.

is required as the first stage in many tissue culture investigations [6-8]. Plant growth regulators (auxin and cytokinin) in the culture media regulate callus development. The concentrations of plant growth regulators might differ based on the plant type. Temperature, light, and other cultural variables all have a role in callus creation and growth. Callus culture can be employed in many experiments and investigations, including protoplast isolation, cell type, cellular selection, somatic embryogenesis, and secondary product synthesis [9].

Trigonella foenum-graecum or fenugreek is a dicotyledonous self-pollinating annual plant belonging to the Fabaceae family Leguminosae.

Fenugreek is one of the most important plants used in medicine and human health since ancient times and in the various civilizations of the world as the Egyptian, Greek, and Indian civilizations have been used in traditional medicine [10, 11]. Fenugreek is grown in many parts of the world, such as North Africa, the middle east, Europe, Southeast Asia, Australia and Argentina. But India is the most productive and consuming country for fenugreek in the world [12]. Since ancient times, fenugreek seeds and aerial parts used in traditional medicine have been a rich protein source, whether for humans or animals Figure 1 [13]. The essential benefits that drew attention to the fenugreek using the plant as a pain reliever, antidiabetic, anti-cancer, heart tonic, increased sexual desire and increased breast milk [14, 15].

In addition, the fenugreek plant contains high levels of saponin, medicinal alkaloids, antioxidant and steroid compounds, and a great source of important plant hormone trigonelline used in the treatment of various types of cancers; many studies prove the importance of trigonelline as a sedative [16]. Fenugreek is characterized by the diversity of medicinal components such as volatile substances, amino acids and phenolic substances and considering an anti-inflammatory plant Figure 1, Figure 2, Figure 3 [17, 18].

Some researchers have documented severe bronchospasms, wheezing, and diarrhoea in a person allergic to curry powder containing fenugreek. According to the findings, patients tested positive for an allergic reaction to the plant extract in a skin patch study. Fenugreek consumption has also been linked to temporary side effects such as diarrhoea, flatulence, and dizziness [19].

There are bioactive elements in fenugreek that may impact human glucose and cholesterol levels, including dietary fibre (galactomannan) and steroid steroidoids (diosgenin). Consumption of fenugreek may cause hypoglycaemia; therefore, patients should have their blood glucose monitored before using fenugreek as a dietary supplement. However, there have been no reports of clinically significant side effects from taking fenugreek as a food or medical complement [20].

Toxic plants may come from various sources, such as pollutants or chemical compounds found in the plant itself. Research into herbal extract potential toxicity uses many tests, including in vivo experiments on laboratory animals and a variety of other biological models.

Alternative biological tests, such as *Artemia salina*, *Thamnocephalus platyurus*, *Artemia urmiana*, and *Artemia franciscana* species, have been used in recent research. These toxicity assays are regarded as a valuable tool for assessing toxicity in the early stages [21]. The Brine Shrimp Assay has been extensively utilised for the last 30 years to examine the toxicity of a broad range of plant products [22].

In this study, seeds of fenugreek were used as explant for callus induction by plant tissue culture technique. The seeds of fenugreek sown in Ms media were supplemented with different concentrations of auxins Naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) and cytokinins 6-Benzylaminopurine BAP singly and combined.

Toxicity measurement by brine shrimp assay was used to examine the aqueous extracts of fenugreek seeds, fenugreek plant and callus induced from plant tissue culture by different concentrations of plant growth regulators. Toxicity was calculated for each extract by measuring the value of lethal concentration LC_{50}.

© 2022 AESS Publications. All Rights Reserved.

Figure 1. Fenugreek seeds.

Figure 2. Fenugreek seeds powder.

© 2022 AESS Publications. All Rights Reserved.

Figure 3. Fenugreek plant.

2. METHODOLOGY

2.1. Materials

2.1.1. Raw Materials

Trigonella foenum-graecum seeds were purchased from Mercearia Neighbourhood grocer at Shah Alam, Selangor, Malaysia. Brine shrimp eggs were purchased from Shopee app. Online platform.

2.1.2. Chemicals

Chemicals used in the experiments are sodium hypochlorite, ethanol, distilled water, MS (Murashige and Skoog medium 1962), naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-Benzylaminopurine (BAP), sucrose, Gelrite agar, thymol.

2.2. In Vitro Callus Induction

2.2.1. Surface Sterilisation and Explant Preparation

Soak the fenugreek purchased seeds before the night of culture and then wash the seeds with running water well for 5 minutes to get rid of dust and impurities on the surface of the seeds. Then fenugreek seeds were sterilised with 5% sodium hypochlorite (v/v) for 20 seconds, followed by consecutive rinsing with sterilized distilled water to remove the effect of sodium hypochlorite. After those seeds were swirled with 70% ethanol (v/v) for three minutes. Sterilized seeds were again rinsed with sterilized distilled water to remove the toxic effect of ethanol. To remove the extra water, the aseptic seeds were put within a Petri dish on sterilised Whatman's filter paper.

2.2.2. Media Preparation and Seeds Culture

MS medium was supplemented with 30 g/L sucrose as an energy source and fortified with various concentrations of NAA, 2,4-D (0.5, 1.0, and 2.0 mg/L), and BAP hormones (1.0, 2.0, and 3.0 mg/L) singly and in combination. Hormones were not used in the control group. Before to autoclaving, the medium was solidified with 4 g/L agar and

© 2022 AESS Publications. All Rights Reserved.

the pH was adjusted to 5.8. The media were autoclaved for 20 minutes at 121°C. After autoclaving, 20 ml of liquid media was poured into pillboxes, which were then allowed to cool and harden before being labelled. The aseptic seeds were then transferred to the hardened MS medium using sterile forceps. Cultured seeds were kept for 30 days, in a culture chamber of a plant tissue culture at 25±2 C in a 16 hours light and 8 hours dark period with relative humidity ranging from 40–60 %. Each treatment included 15 pillbox replicas, each implanted with three seeds. Based on the data, the optimal hormone concentrations for callus induction were found during this period (frequency of callus induction, callus index, callus morphology colour and texture). The optimal hormone concentration for in vitro callus induction was determined by taking the final mean of all the frequency and index of callus data after 30 days. After 30 days, all callus induced by all treatments were collected and stored to create the aqueous callus extract for the brine shrimp assay.

2.3. Extract Preparation

2.3.1. Aqueous Seed Extract Preparation

10 grams of fenugreek seeds were weighed. Then the seeds were ground by the mill and powdered. Following, the grinded powder was soaked in 100ml of distilled water for 24 hours. After that, filtered the extract with muslin cotton and filtered with Whatman No. 1 filter paper [23, 24] prepared a stock solution of extract 1 mg/ml and kept at 4 °C for further usage.

2.3.2. Aqueous Fenugreek Plant Extract Preparation

Fenugreek plant was grown for 30 days in an outdoor environment. Afterwards, 10 grams of Fenugreek plant was weighed, and samples were washed using tap water to eliminate dust and dirt particles. Then the plant was placed in the electric blender to make the plant pulp mixture. Distilled water was added during the blending to facilitate the filtration processes (using muslin cotton and filter paper Whatman No. 1). Eventually, 1 mg/ml of stock solution were prepared and kept at 4 °C for further usage.

2.3.3. Fenugreek Callus Extract Preparation

Callus from all treatments were collected and 10 gm of fenugreek seeds callus were grinded using mortar and pestle and added with water. The extracts were filtered using Whatman No. 1 filter paper [25] Stock solution 1 mg/ml were prepared and kept at 4 °C for further using.

2.4. Toxicity Analysis

2.4.1. Brine Shrimp Hatching

1 litre of distilled water was poured it into a homemade brain shrimp hatcher. 35 grams of NaCl was added into the water-filled hatcher. Adequate aeration was maintained inside the hatcher using air pump. roughly 1 g of brine shrimp eggs was added into the jar's and stir with the water. The nauplii hatches in 20-24 hours. After the following 24 hours, the hatched nauplii were separated from the empty egg and shells residues.

2.4.2. Toxicity test

Prepare Petri dishes to contain 20 ml of artificial seawater. Added different concentrations of the stock solution to the petri dishes and make the dilution with the seawater (5ml, 4ml, 3ml, 2ml, 1ml) will get five different concentrations (250 µg/ml, 200 µg/ml, 150 µg/ml, 100 µg/ml, 50 µg/ml) with five replicates for each concentration according to procedures described by Meyer study with simple modified [26, 27].

Experiment with different concentrations of the plant extracts on the nauplii. The experiment was conducted in triplicate to ensure that the data obtained were statistically repeatable [22]. After 24 hours, survivors were counted, and percentage of death (mortality) was determined using following equation:

© 2022 AESS Publications. All Rights Reserved.

$$\% \text{ Mortality} = \frac{\text{number of dead nauplii}}{\text{number of dead nauplii} + \text{number of live nauplii}} \times 100$$

The lack of controlled forward movement during 30 seconds of observation is defined as the bioassay's death endpoint. The mortality percentage of the nauplii was determined for each concentration and control. The number of deaths for each petri dish was calculated by counting the number of dead and living naupliis.

2.4.3. LC_{50} Calculate

The toxicity of herbal extracts was typically valued as LC_{50} values when compared to Meyer's or Clarkson's toxicity indexes. Using probit analysis, the lethality concentration (LC_{50}) was determined using following equation.

$$Y = ax + b$$

Replace Y value to LC_{50} by using probit regression and it is equal to 5.

Where a = X variable, b = intercept value.

The LC_{50} (median lethal concentration) values were derived by graphing the log concentration against the death percentage on a probit scale by Finney [28] and utilizing the regression line produced [28].

2.5. Statistical Analysis

SPSS (Version 17. O) software was used for descriptive statistical analysis, and data were analysed using one-way ANOVA. Tukey's test was used to separate the means. For analysis data of LC_{50} used regression on the Excel Platform.

3. RESULTS AND DISCUSSION

3.1. Callus Induction

T. foenum-graecum seeds induced callus in MS (Murashige and Skoog) medium supplemented with various doses of the hormones BAP, NAA, and 2,4-D after 30 days.

3.1.1. Effect of Different Concentrations of BAP on Callus Induction of T. Foenum-Graecum Seeds

As shown in Table 1, there is no effect of BAP hormone at all on induced callus of *T. foenum-graecum* seeds after 30 days of culture. These findings reinforce the general belief that the cytokinin hormones are plant growth regulators that influence plant growth and development, establishing blooms and promoting fruit richness by encouraging cell division. At the same time, it does not affect or induce the plant to form callus [29].

3.1.2. Effect of Different Concentrations of NAA on Callus Induction of T. Foenum-Graecum Seeds

As shown in Table 1, and Table 2, NAA hormone leads to induce callus of *T. foenum-graecum* from seeds. All concentrations of NAA hormone (0.5 mg/l, 1.0 mg/l 2.0 mg/l) used in this study caused to induce the callus after 30 days of culture for explant *T. Foenum-graecum* seeds in MS media supplemented with previous concentrations of NAA hormone. The callus was successfully induced in all different concentrations of NAA hormone. No callus induction was observed in the absence of NAA hormone in Ms media which was used as control.

These outcomes agreed with Mawahib, et al. [30], who studied auxins' effect on callus induction using *T. Foenum-graecum* as an explant. The results in Table 1 demonstrated that raising NAA hormone concentrations to 2.0 mg/l resulted in a significant rise ($P \leq 0.05$) in callus index, which then began to decrease when the hormone concentration was reduced to 1.0 mg/l.

© 2022 AESS Publications. All Rights Reserved.

Table 1. Effect of different concentrations of BAP, NAA and 2,4-D on callus induction of *T. foenum-graecum* frequency of callus, callus weight, and callus index. Data represent mean ± SE.

Treatments	Hormone Mg/L				Mean±SE	
	BAP	NAA	2,4-D	Callus %	Callus Weight (g)	Callus Index
MSO	0	0	0	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T1	1	0	0	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T2	1	0.5	0	100^{a}	0.37 ± 0.02^{bc}	37 ± 2.04^{b}
T3	1	1	0	93.33^{a}	0.22 ± 0.05^{d}	21 ± 5.88^{c}
T4	1	2	0	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T5	1	0	0.5	33.33^{b}	0.03 ± 0.01^{ef}	0.85 ± 0.10^{de}
T6	1	0	1	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T7	1	0	2	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T8	2	0	0	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T9	2	0.5	0	100^{a}	0.50 ± 0.06^{ab}	49.33 ± 6.96^{ab}
T10	2	1	0	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T11	2	2	0	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T12	2	0	0.5	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T13	2	0	1	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T14	2	0	2	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T15	4	0	0	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T16	4	0.5	0	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T17	4	1	0	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T18	4	2	0	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T19	4	0	0.5	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T20	4	0	1	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T21	4	0	2	0^{c}	0 ± 0^{f}	0 ± 0^{e}
T22	0	0.5	0	100^{a}	0.15 ± 0.01^{de}	15 ± 0.74^{cd}
T23	0	1	0	93.33^{a}	0.13 ± 0.01^{def}	13 ± 1.95^{cde}
T24	0	2	0	100^{a}	0.16 ± 0.02^{de}	16 ± 2.45^{cd}
T25	0	0	0.5	100^{a}	0.40 ± 0.05^{cd}	40 ± 5.36^{ab}
T26	0	0	1	100^{a}	0.52 ± 0.08^{a}	52 ± 8.24^{a}
T27	0	0	2	100^{a}	0.51 ± 0.04^{a}	51 ± 4.02^{ab}

Note: a, b, c, d, e and f show vertically mean there is a significant difference at $p<0.05$ significant level.

Table 1 shows the effect of this hormone, NAA. Promoted NAA hormone concentrations in the MS medium increased callus formation. The greatest mean of callusing index and proliferation from *T. Foenum-graecum* seeds was (16±4.90), with 100% callus induction in the 30 days by 2.0mg/l NAA. Followed by 0.5 mg/l since was the callus index (15±1.49), with 100% callus induction in 30 days of culture. The lowest callus induction observed in concentration 1 mg/l of NAA hormone where was the callus index (13±4.30) and callus induction percentage 93.33% after 30 days of culture. The texture of induced callus of *T. Foenum-graecum* was soft and showed white colour in all concentrations used in these treatments as stated in Table 2.

These findings are in line with those reported by Elnour et al., whom employed hypocotyls and cotyledons of *T. Foenum-graecum* as explants instead of seeds [31] Callus weight that induced in concentrations (0.5 mg/l, 1.0 mg/l, 2.0 mg/l) of NAA hormone showed differences where was (0.15±0.05 gm, 0.13±0.06 gm, 0.16±0.06gm) respectively. The best yield of callus obtained of concentration 2 mg/l of NAA hormone.

3.1.3. Effect of Different Concentrations of 2,4-D on Callus Induction of T. Foenum-Graecum Seeds

As seen in Table 1, and Table 2, using 2,4-D hormones with different concentrations (0.5 mg/l, 1.0 mg/l, 2.0 mg/l) to induce callus of *T. Foenum-graecum* seeds produced the better result in this study. The callus was successfully induced in all different concentrations of 2,4-D hormone. No callus induction was observed in the absence of 2,4-D hormone where used the Ms media was without any hormones as control. The results revealed that elevating of 2,4-D hormone concentrations to 1 mg/l improved the callus index significantly ($P<0.05$), whereas increasing up to 2mg/l lowered the callus index and percentage of callus. The greatest mean of callusing index and proliferation from

© 2022 AESS Publications. All Rights Reserved.

T. Foenum-graecum seeds was (52±9.5), with 100% callus induction in the 30 days by 1.0mg/l 2,4-D. Followed by 0.5 mg/l since was the callus index (51±7.05), with 100% callus induction in 30 days of culture.

Table 2. Effect of different concentrations of BAP, NAA and 2,4-D on morphology of callus of *T. foenum-graecum* texture and color.

Treatments	Hormone MG/L				
	BAP	NAA	2,4-D	Callus Texture	Callus Color
MSO	0	0	0	-	-
T1	1	0	0	-	-
T2	1	0.5	0	Soft Callus	Greenish Callus
T3	1	1	0	Soft Callus	Greenish Callus
T4	1	2	0	-	-
T5	1	0	0.5	Soft Callus	White Callus
T6	1	0	1	-	-
T7	1	0	2	-	-
T8	2	0	0	-	-
T9	2	0.5	0	Soft Callus	Greenish Callus
T10	2	1	0	-	-
T11	2	2	0	-	-
T12	2	0	0.5	-	-
T13	2	0	1	-	-
T14	2	0	2	-	-
T15	4	0	0	-	-
T16	4	0.5	0	-	-
T17	4	1	0	-	-
T18	4	2	0	-	-
T19	4	0	0.5	-	-
T20	4	0	1	-	-
T21	4	0	2	-	-
T22	0	0.5	0	Soft Callus	White Callus
T23	0	1	0	Soft Callus	White Callus
T24	0	2	0	Soft Callus	White Callus
T25	0	0	0.5	Soft Callus	Greenish Callus
T26	0	0	1	Soft Callus	Greenish Callus
T27	0	0	2	Soft Callus	Brownish Callus

This study agrees with Elnour, et al. [31], where used 2,4-D hormone for callus induction of *T. foenum-graecum* using hypocotyls and cotyledon as explant. The outcome showed that 2,4-D hormone gave high frequency of callus induction. As well as in line with a study by Abd Elaleem, et al. [32] since using 2,4-D hormone in callus induction and the callus index increase from 0.5 mg/l, 1 mg/l, 1.5 mg/l, while decreased when elevated to 2 mg/l in hypocotyl explant. Table 1, shows the effect of this hormone, 2,4-D. Promoted 2,4-D hormone concentration of 2,4-D to 2 mg/l. As well callus frequency showed 100% in all concentrations. Callus weight that induced in concentrations (0.5 mg/l, 1 mg/l, 2 mg/l) of 2,4-D hormone showed difference where was (0.40±0.09 gm, 0.52±0.17 gm, 0.51±0.12 gm) respectively. Best yield of callus obtained of concentration 1 mg/l of 2,4-D hormone. Texture of induced callus of *T. Foenum-graecum* was soft and showed white colour in all concentrations used in these treatments as stated in Table 2 and Figure 4.

3.1.4. Effect of Different Concentrations of BAP with NAA on Callus Induction of T. Foenum-Graecum Seeds

Callus formation was seen in 3 treatments out of the 9 treatments from seed explants grown on MS medium supplemented with varying concentrations of BAP and NAA. As seen in Table 1 and Table 2, Maximum callus induction was seen after 30 days of cultures on MS medium supplemented with 2.0 mg/l BAP with 0.5 mg/l NAA, followed by 1.0 mg/l BAP with 0.5 mg/l NAA, and the lowest callus induced from 1.0 mg/l BAP with 1 mg/l NAA, where the callus index was 49.33±5, 37±0 4.05, and 21±10 respectively. As for the other concentrations, the absence

© 2022 AESS Publications. All Rights Reserved.

of callus was observed and in the MSO used as a control in this study. It was noted that the high concentrations of BAP with NAA gave negative results and didn't show any response in callus formation.

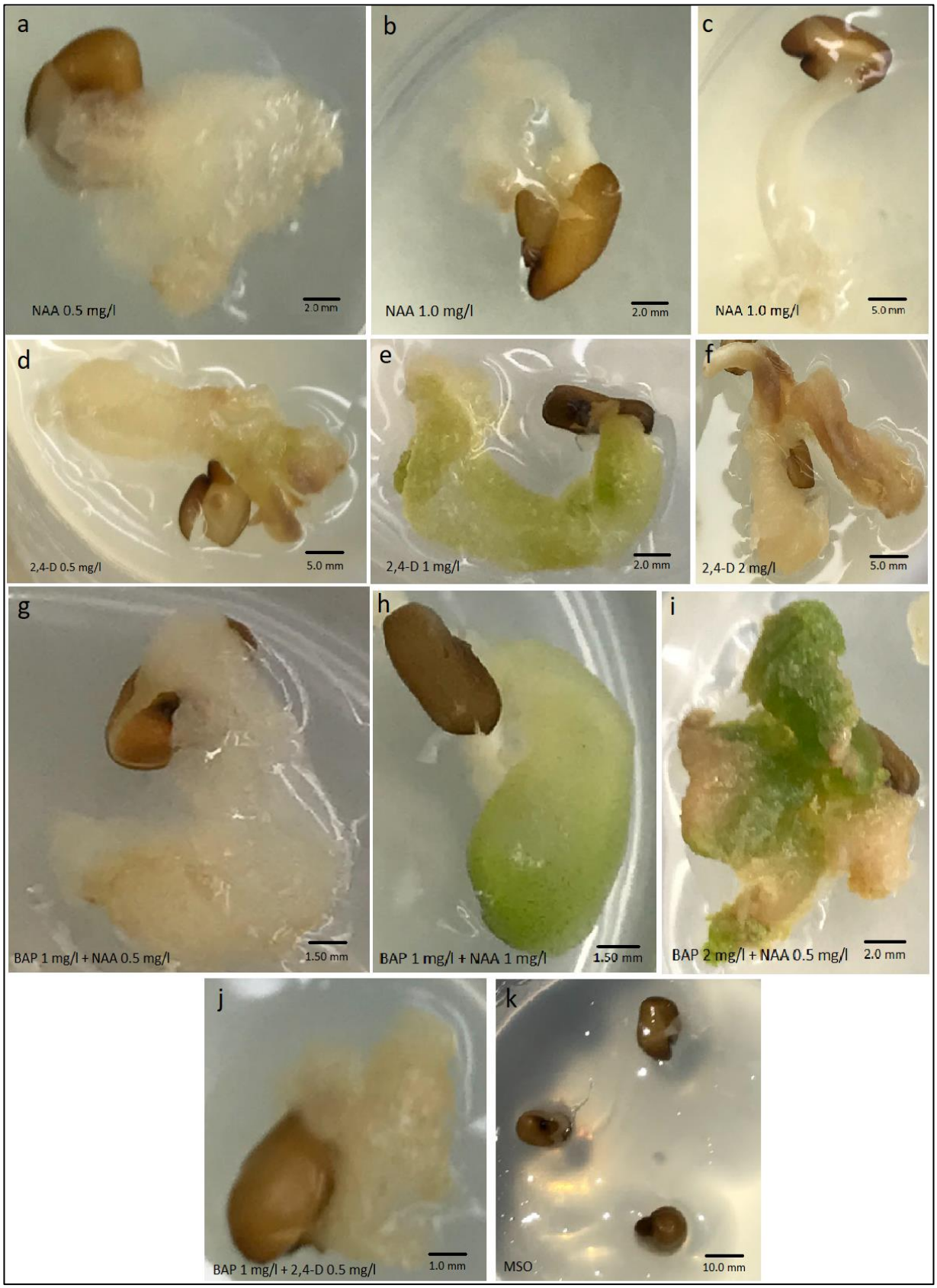

Figure 4. Callus induction of *T. foenum-graecum* Seeds by different concentrations of BAP, NAA and 2,4-D.

© 2022 AESS Publications. All Rights Reserved.

The frequency of callus induction for this study appeared 100% for 2.0 mg/l BAP with 0.5 mg/l NAA and 1.0 mg/l BAP with 0.5 mg/l NAA. The results of this study showed some improvement over other studies such as Raveesha and Ashalatha [25], which used *D. arayalpathra* as an explant, where the frequency of callus induction to concentrations 1.0 mg/l BAP with 0.5 mg/l NAA and 2.0 mg/l with 0.5 mg/l NAA was 96.67 and 95 respectively. The result of this study is in line with the study conducted by Bakar, et al. [33] since *Celosia argentea* was used as explant, the ratio of callus induction was 98% for 1.0 mg/l BAP with 0.5 mg/l NAA and 1 mg/l BAP with 1.0 mg/l NAA which shows it is almost same with this result.

The weight of the induced callus from the concentrations that gave a positive response differed where the highest callus weight was (0.50±0.10 gm) at the concentration 2.0 mg/l BAP with 0.5 mg/l NAA. Followed by (0.37±0.24 gm) at the concentration 1 mg/l BAP with 0.5 mg/l NAA.

While yield of callus weight was (0.22±0.10 gm) the lowest at 1.0 mg/l BAP with 1.0 mg/l NAA and the lowest frequency of callus induction 93.33 %. Texture of induced callus of *T. Foenum-graecum* was soft and showed green colour in all concentrations used in these treatments as stated in Table 2 and Figure 4.

3.1.5. Effect of Different Concentrations of BAP with 2,4-D on Callus Induction of T. Foenum-Graecum Seeds

As shown Table 1 and Table 2, the combination of BAP hormone and 2,4-D did not show any obvious induction of callus of *T. Foenum-graecum* seeds except for the concentration 1.0 mg/l of BAP with 0.5 mg/l of 2,4-D. This concentration showed a weak result and caused the induction of callus in a low percentage 33.33% only, as well as the weight of callus was low (0.03±0.01 gm). The callus index was (0.85±0.12) and the texture was soft and white colour Figure 4.

Overall, on 1.0 mg/l and 2.0 mg/l 2,4-D respectively, the maximum callus induction was found (52±9.5 gm), (0.51±0.12 gm). When compared to other treatments, the hormone in 30 days was significant ($p \leq 0.05$). It was observed that the kind and concentration of growth regulators had a significant influence on callus induction of *T. Foenum-graecum* seeds. Followed by a combination of BAP and NAA since 2 mg/l BAP with 0.5 mg/l NAA callus induction was found (0.50±0.10 g) and 1 mg/l of BAP with 0.5 mg/l NAA gave (0.37±0.24 g) and a minimum yield of this combination found on 1 mg/l BAP with 1mg/l NAA (0.22±0.10 g).

Using the NAA hormone alone with different concentrations (0.5 mg/l, 1.0 mg/l 2.0 mg/l) also showed some effect of callus induction of *T. Foenum-graecum* seeds where gave yield of callus (0.15±0.05 g, 0.13±0.06 g, 0.16±0.06 g) respectively. While the combination of BAP with 2,4-D showed the lowest yield of callus on concentration 1mg/l BAP with 0.5 mg/l 2,4-D (0.03±0.01 g) and no effect at all for other concentrations. The hormones in 30 days were significant ($p \leq 0.05$).

3.2. Assessment of T. Foenum-graecum Extracts Toxicity by Brine Shrimp Lethality Assay

The brine shrimp lethality assay (BSLA) is a low-cost and straightforward bioassay for determining the effectiveness of phytochemicals found in plant extracts. According to the findings of this investigation, the level of lethality was directly related to the concentration of the extract.

3.2.1. Toxicity of Aqueous Extract of T. Foenum-Graecum Seeds

The percentage of A. salina nauplii mortality increased as solution concentration was increased. After 24 hours of monitoring, all the shrimp nauplii in the distilled water (negative control) survived. Whereas in the Thymol (positive control), all the naupliis died within 24 hours in concentrations 150 µg/ml, 200 µg/ml and 250 µg/ml Figure 5. The highest mortality percentage was observed at a concentration 250 µg/ml of *T. Foenum-graecum* seeds aqueous extract 26.60 %. In comparison, the lowest mortality rate was observed at a concentration 50 µg/ml 6.66 % as shown in Figure 6.

© 2022 AESS Publications. All Rights Reserved.

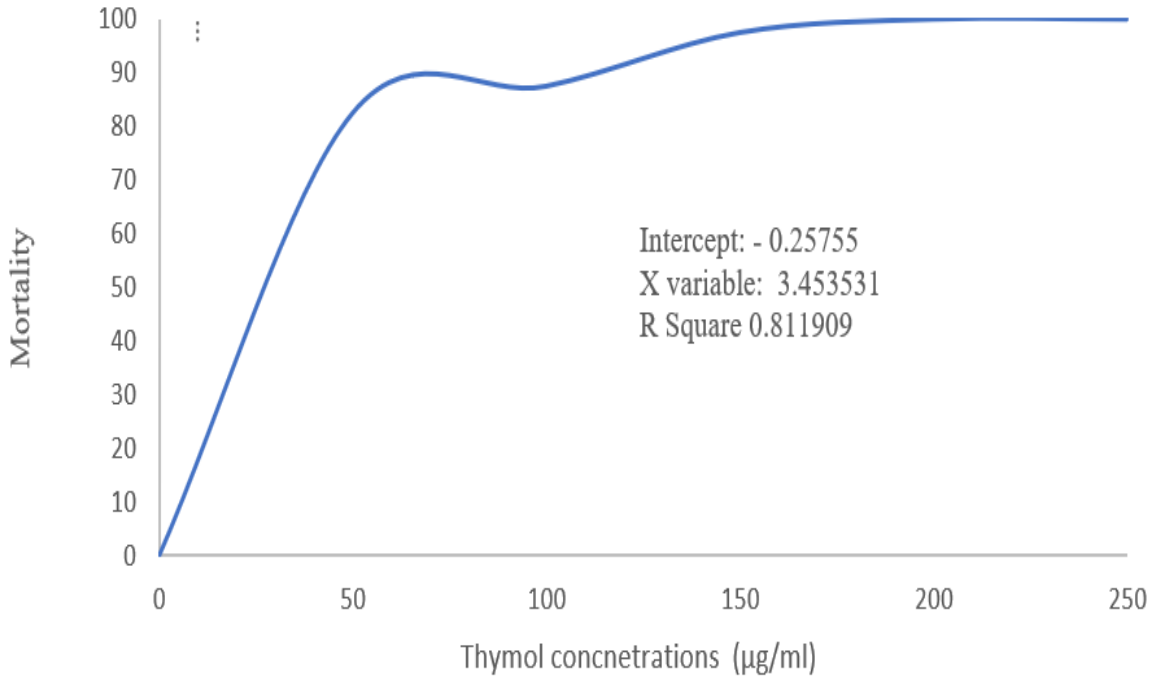

Figure 5. Relationship between the mortality percentage and increased thymol concentrations as positive control.

The lethality of an aqueous extract of *T. Foenum-graecum* seeds on brine shrimps naupliis was determined Table 3 LC_{50} 954.99 µg/ml. This value of LC_{50} is considered low toxic according to Clarkson's toxicity criterion [34]. Fenugreek seeds contain many active compounds that may be reasons for the low toxicity at high doses, such as flavonoids, alkaloids, coumarins, saponins, and other antioxidants [35]. Fenugreek seeds also had high levels of the toxic metals Cd and Pb, making the fenugreek seeds unsuitable for daily consumption [36]. Therefore these substances might how to cause toxicity against brine shrimp naupliis.

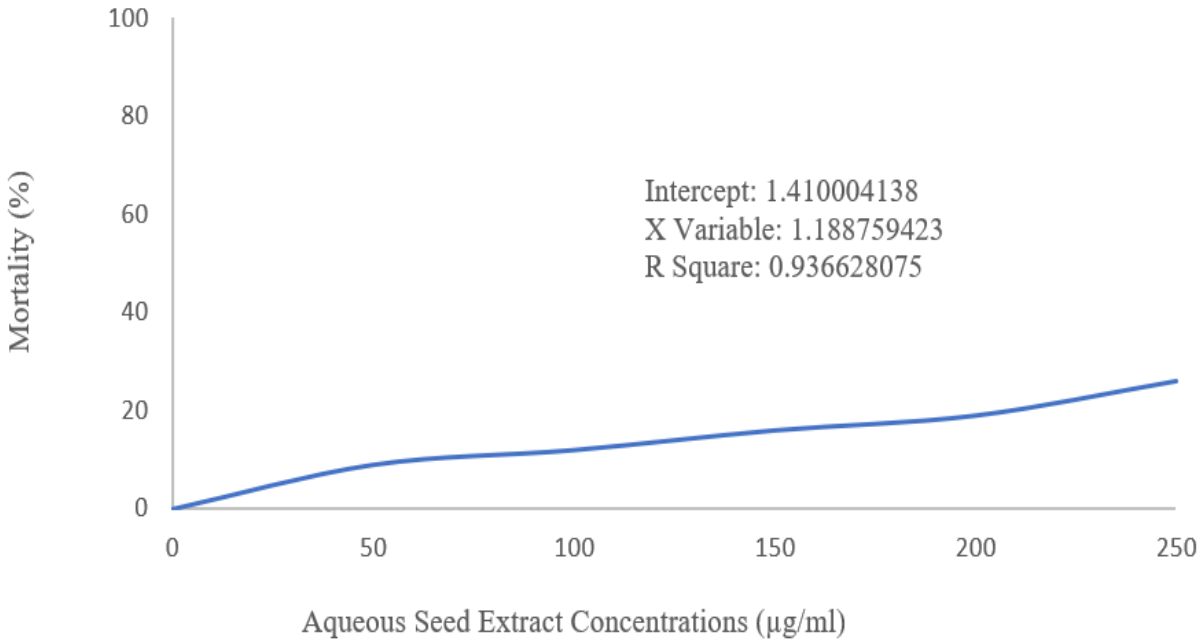

Figure 6. Relationship between the mortality percentage and increased *T. foenum-graecum* seeds extract concentrations.

© 2022 AESS Publications. All Rights Reserved.

Table 3. Mortality percentage of shrimp Nauplii and LC_{50} after treating with aqueous extract of *Trigonella foenum-graecum* seeds, plant, and callus and toxicity classification according to Clarkson's toxicity criterion.

Extracts	Concentrations µg/ml	No. of Surviving Nauplii (After 24 Hours)			Total No. of Nauplii Survivors	% Mortality	LC_{50} µg/ml	Toxicity Class
		T1	T2	T3				
Thymol (positive control)	50	0	0	1	1	97.00%	30.33	High toxic
	100	1	0	0	1	97.00%		
	150	0	0	0	0	100.00%		
	200	0	0	0	0	100.00%		
	250	0	0	0	0	100.00%		
Distilled water (negative control)	50	10	10	10	30	0.00%	-	Non toxic
	100	10	10	10	30	0.00%		
	150	10	10	10	30	0.00%		
	200	10	10	10	30	0.00%		
	250	10	10	10	30	0.00%		
TFG Seed Extract	50	9	10	9	28	6.66%	954.99	Low Toxic
	100	9	9	9	27	10%		
	150	8	9	9	26	13.30%		
	200	8	8	8	24	20%		
	250	7	8	7	22	26.60%		
TFG Whole Plant Extract	50	9	10	10	29	3.33%	1237.98	Non toxic
	100	9	9	10	28	6.66		
	150	8	9	10	27	10%		
	200	9	7	9	25	16.66%		
	250	7	8	8	23	23.33%		
TFG Callus Extract	50	9	10	10	29	3.33%	1801.52	Non toxic
	100	10	9	19	29	3.33%		
	150	9	9	10	28	6.66%		
	200	7	10	9	26	13.33%		
	250	8	9	8	25	16.66%		

3.2.2. Toxicity of Aqueous Extract of T. Foenum-Graecum Plant

The percentage of A. salina nauplii mortality increased as solution concentration was increased. After 24 hours of monitoring, all the *A. salina* naupliis in the distilled water (negative control) survived. Whereas in the Thymol (positive control), all the naupliis died within 24 hours in concentrations 150 µg/ml, 200 µg/ml and 250 µg/ml. The highest mortality percentage was observed at a concentration 250 µg/ml of *T. Foenum-graecum* plant aqueous extract 23.33 %. In comparison, the lowest mortality rate was observed at a concentration 50 µg/ml 6.66 % as shown in Figure 7. The maximum concentration of plant extract in this study gave less mortality than the same concentration for the seeds, where was 26.60 %.

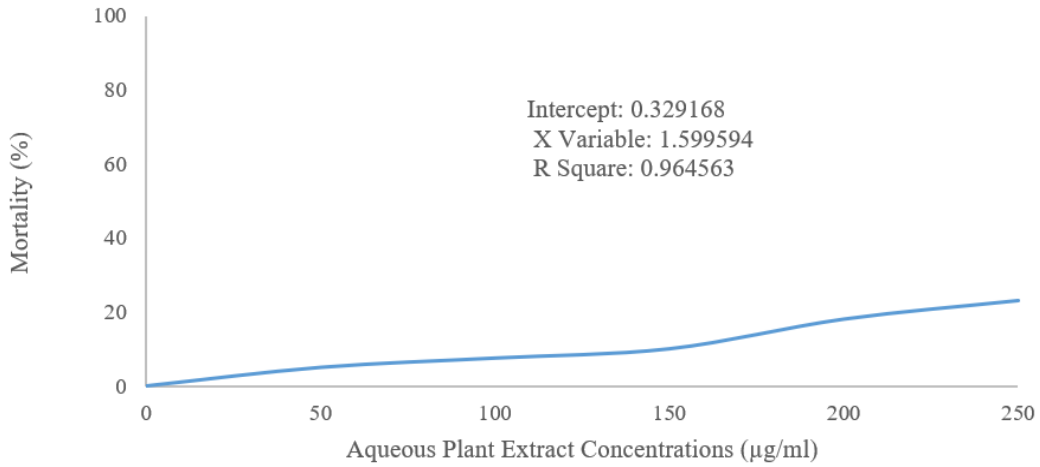

Figure 7. Relationship between the mortality percentage and increased *T. foenum-graecum* plant extract concentrations.

© 2022 AESS Publications. All Rights Reserved.

As shown in Table 3, the LC_{50} value of whole plant extract was 1237.98 µg/ml. according to Clarkson's toxicity criterion *T. Foenum-graecum* whole plant extract considers non-toxic. The explanation of this value compared to seeds LC_{50} 954.99 µg/ml value is the seeds contain high concentration of flavonoids, alkaloids, coumarins, saponins that caused toxicity in the seeds, while the percentages of these substances lower in the whole plant.

3.2.3. Toxicity of Aqueous Extract of T. Foenum-Graecum Callus

The mortality of *A. salina* nauplii after 24 hours of exposure to various concentrations of *T. foenum-graecum* callus was same at the concentrations 50 µg/ml, 100 µg/ml 3.33% Table 3. after increased the concentration of callus to 150 µg/ml, the mortality increased to 6.66%. The highest percentage of mortality was found at concentration 250 µg/ml 16.66%. The mortality percentages also increased with elevating the concentrations of callus extract Figure 8. Clarkson's toxicity criterion indicated that the callus extract was considered non-toxic, since the LC50 was 1801.52 µg/ml. This value of LC50 was the highest among the rest of extracts plant and seeds in this study. Therefor the callus extract of *T. foenum-graecum* considered less toxicity than others in this study [34].

These findings reinforce the general belief that the callus of plant contain small amounts of plant components since the callus is undifferentiated cells, and the percentage of plant specific compounds is low. These results are in line with the study demonstrated on mice by using callus of Pulicaria incisa. That study approved no toxicity of callus extract on the mice when treating the mice with different concentrations of callus extract of *Pulicaria incisa* [37].

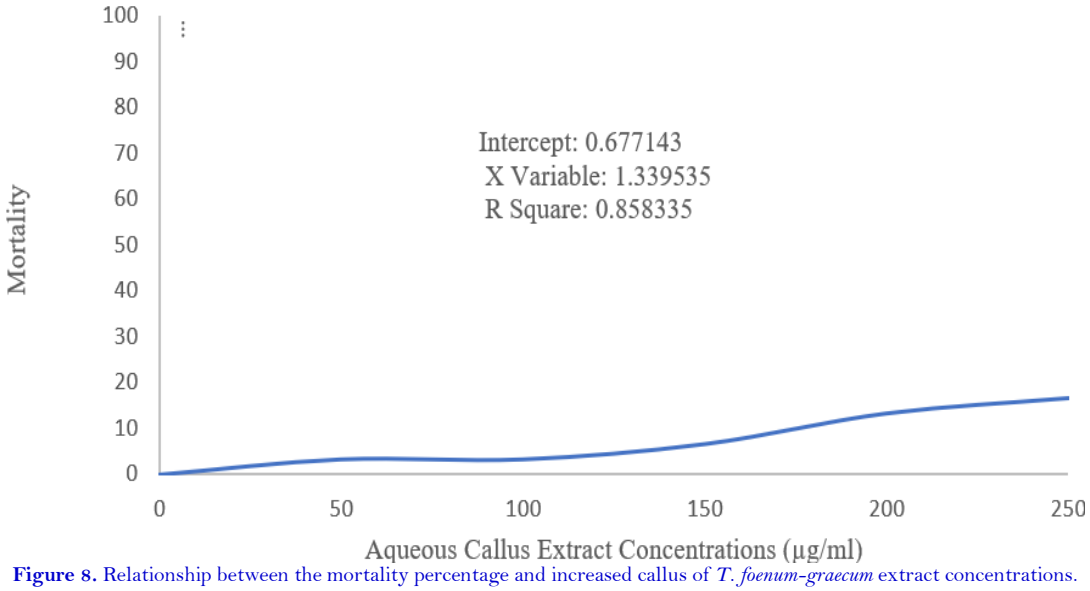

Figure 8. Relationship between the mortality percentage and increased callus of *T. foenum-graecum* extract concentrations.

4. CONCLUSION AND RECOMMENDATIONS

This research investigated the effect of single and combined hormones on induce callus of *T. foenum-graecum* seeds. The best yield of callus appeared during 30 days on concentration 1 mg/l and 2 mg/l of 2,4-D, and 2 mg/l of BAP combined with 0.5mg/l NAA, the callus index was 52±9.5%, 51±7.05 and 49.33±5 respectively. The callus weight for those hormones was 0.52±0.17 gm, 0.51±0.12 gm and 0.50±0.10 gm.

The second part of this research focused on studying the toxicity of *T. foenum-graecum* using the brine shrimp assay, where the study relied on three different types of *T. foenum-graecum* extracts. *The first part of the study obtained T. foenum-graecum seeds aqueous extract, whole plant aqueous extract, and aqueous callus extract.* The study showed the result of the toxicity examination, as the highest toxicity was in the aqueous extract of fenugreek seeds, followed by the toxicity of the whole plant. The Least toxicity appeared in aqueous callus extract since Lc_{50} was 954.99 µg/ml, 1237.98µg/ml, 1801.52 µg/ml, respectively.

© 2022 AESS Publications. All Rights Reserved.

This study opens the window to study the effect of other hormones on germination and induction of callus *T. foenum-graecum* in vitro. And try to use hormones in different treatments and more extended incubation periods. As well as recommend using other types of auxin to get a better callus yield. Also, this window study opened opportunities to study the toxicity of fenugreek using different solvents such as methanol, ethanol and acetone in brine shrimp assay.

Funding: This research is supported by Universiti Teknologi Mara, Malaysia (Grant numbers: MOHE-(600-RMI/FRGS 5/3(49/2015)) and, UiTM Grant 600-IRMI 5/3/GIP (024/2018).).
Competing Interests: The authors declare that they have no competing interests.
Authors' Contributions: All authors contributed equally to the conception and design of the study.

REFERENCES

[1] S. Gonçalves and A. Romano, "Production of plant secondary metabolites by using biotechnological tools," Secondary metabolites, sources and applications," *IntechOpen*, pp. 81-99, 2018.Available at: https://doi.org/10.5772/intechopen.76414.

[2] A. Lubbe and R. Verpoorte, "Cultivation of medicinal and aromatic plants for specialty industrial materials," *Industrial Crops and Products*, vol. 34, pp. 785-801, 2011.Available at: https://doi.org/10.1016/j.indcrop.2011.01.019.

[3] M. I. Dias, M. J. Sousa, R. C. Alves, and I. C. Ferreira, "Exploring plant tissue culture to improve the production of phenolic compounds: A review," *Industrial Crops and Products*, vol. 82, pp. 9-22, 2016.Available at: https://doi.org/10.1016/j.indcrop.2015.12.016.

[4] N. Kapoor, A. Nigam, S. S. Hasan, and S. Malhotra, *Block-3 environmental issues and concerns*. New Delhi: Indira Gandhi National Open University, 2019.

[5] J. Coleman, D. Evans, and A. Kearns, *Plant cell culture*. London: Taylor & Francis, 2020.

[6] M. Orgeç, S. K. Verma, G. Sahin, N. Zencirci, and E. Gürel, "In vitro tissue culture protocol of ancient einkorn (Triticum monococcum ssp. monococum) wheat via indirect shoot regeneration," *In Vitro Cellular & Developmental Biology-Plant*, vol. 57, pp. 143-151, 2021.Available at: https://doi.org/10.1007/s11627-020-10122-8.

[7] B. M. Twaij, Z. H. Jazar, and M. N. Hasan, "Trends in the use of tissue culture, applications and future aspects," *International Journal of Plant Biology*, vol. 11, pp. 1-14, 2020.Available at: https://doi.org/10.4081/pb.2020.8385.

[8] H. H. Mostafa, H. Wang, J. Song, and X. Li, "Effects of genotypes and explants on garlic callus production and endogenous hormones," *Scientific Reports*, vol. 10, pp. 1-11, 2020.Available at: https://doi.org/10.1038/s41598-020-61564-4.

[9] S. Ashokhan, R. Othman, M. H. Abd Rahim, S. A. Karsani, and J. S. Yaacob, "Effect of plant growth regulators on coloured callus formation and accumulation of azadirachtin, an essential biopesticide in Azadirachta indica," *Plants*, vol. 9, pp. 1-17, 2020.Available at: https://doi.org/10.3390/plants9030352.

[10] S. I. Abdel-Wahab and E. I. Abdel-Wahab, "Cropping systems of fenugreek with faba bean to reduce broomrape infestation," *Legume Research: An International Journal*, vol. 44, pp. 579-592, 2021.Available at: https://doi.org/10.18805/lr-621.

[11] A. D. Kandhare, S. L. Bodhankar, V. Mohan, and P. A. Thakurdesai, "Prophylactic efficacy and possible mechanisms of oligosaccharides based standardized fenugreek seed extract on high-fat diet-induced insulin resistance in C57BL/6 mice," *Journal of Applied Pharmaceutical Science*, vol. 5, pp. 35-45, 2015.

[12] P. Zandi, S. K. Basu, W. Cetzal-Ix, M. Kordrostami, S. K. Chalaras, and L. B. Khatibai, "Fenugreek (Trigonella foenum-graecum L.): An important medicinal and aromatic crop," *Active Ingredients from Aromatic and Medicinal Plants*, pp. 207-224, 2017.Available at: http://dx.doi.org/10.5772/66506.

[13] M. H. Shahrajabian, W. Sun, A. Magadlela, S. Hong, and Q. Cheng, *Fenugreek cultivation in the middle east and other parts of the world with emphasis on historical aspects and its uses in traditional medicine and modern pharmaceutical science," in Fenugreek*. Singapore: Springer, 2021.

© 2022 AESS Publications. All Rights Reserved.

[14] M. T. Salman and F. Qadeer, *Pharmacological actions and therapeutic potential of trigonella foenum-graecum L.," in Fenugreek.* Singapore: Springer, 2021.

[15] Y. A. Bin Jardan, A. Ahad, M. Raish, M. A. Alam, A. M. Al-Mohizea, and F. I. Al-Jenoobi, "Effects of garden cress, fenugreek and black seed on the pharmacodynamics of metoprolol: An herb-drug interaction study in rats with hypertension," *Pharmaceutical Biology*, vol. 59, pp. 1088-1097, 2021.Available at: https://doi.org/10.1080/13880209.2021.1961817.

[16] M. Bahmani, H. Shirzad, M. Mirhosseini, A. Mesripour, and M. Rafieian-Kopaei, "A review on ethnobotanical and therapeutic uses of fenugreek (Trigonella foenum-graceum L)," *Journal of Evidence-Based Complementary and Alternative Medicine*, vol. 21, pp. 53-62, 2016.Available at: https://doi.org/10.1177/2156587215583405.

[17] Z. Moradi and J. B. Zadeh, "Fenugreek (Trigonella foenum-graecum L .) as a valuable medicinal plant fenugreek (Trigonella foenum-graecum L .) As a Valuable Medicinal Plant," no. March," *International Journal of Advanced Biological and Biomedical Research*, vol. 1, pp. 922-931, 2015.

[18] J. W. Kimondo, "An evaluation of antioxidant and anti-inflammatory effects of natural foods and medicinal plants of the ilkisonko maasai community, Kenya," Doctoral Dissertation, University of Nairobi, 2020.

[19] J. E. Thomas, M. Bandara, D. Driedger, and E. L. Lee, "Fenugreek in western Canada," *American Journal of Plant Sciences Biotech*, vol. 5, pp. 32-44, 2011.

[20] A. M. Mortazavian, N. Khorshidian, M. Yousefi Asli, M. Arab, and A. Adeli Mirzaie, "Fenugreek: Potential applications as a functional food and nutraceutical," *Nutrition and Food Sciences Research*, vol. 3, pp. 5-16, 2016.

[21] V. Thangapandi and T. Pushpanathan, "Comparison of the Artemia salina and Artemia fransiscana bioassays for toxicity of Indian medicinal plants," *Journal of Coastal Life Medicine*, vol. 2, pp. 453-457, 2014.Available at: https://doi.org/10.12980/jclm.2.201414j29.

[22] N. Sharma, P. C. Gupta, A. Singh, and C. Rao, "Brine shrimp bioassay of Pentapetes phoenicea Linn. and Ipomoea carnea jacq. leaves," *Der Pharmacia Lettre*, vol. 5, pp. 162-167, 2013.

[23] A. A. Allaq, N. J. Sidik, A. Abdul-Aziz, and I. A. Ahmed, "Antioxidant, antibacterial, and phytochemical screening of ethanolic crude extracts of Libyan Peganum harmala seeds," *Journal of Pharmaceutical Research International*, vol. 33, pp. 74-82, 2021.

[24] H. Meguellati, S. Ouafi, S. Saad, and N. Djemouai, "Evaluation of acute, subacute oral toxicity and wound healing activity of mother plant and callus of Teucrium polium L. subsp. geyrii Maire from Algeria," *South African Journal of Botany*, vol. 127, pp. 25-34, 2019.Available at: https://doi.org/10.1016/j.sajb.2019.08.023.

[25] H. R. Raveesha and K. S. Ashalatha, "Callus Induction, phytochemical studies and antibacterial activity of decalepis arayalpathra (Joseph and Chandras) Venter," *International Journal of Pharmacy and Pharmaceutical Sciences*, vol. 9, pp. 136-140, 2017.

[26] N. M. A. Shaala, S. Z. Zulkifli, A. Ismail, M. N. A. Azmai, and F. Mohamat-Yusuff, "Lethal concentration 50 (LC50) and effects of Diuron on morphology of brine shrimp Artemia salina (Branchiopoda: Anostraca) Nauplii," *Procedia Environmental Sciences*, vol. 30, pp. 279-284, 2015.Available at: https://doi.org/10.1016/j.proenv.2015.10.050.

[27] B. N. Meyer, N. R. Ferrigni, J. E. Putnam, L. B. Jacobsen, D. E. Nichols, and J. L. McLaughlin, "Brine shrimp: A convenient general bioassay for active plant constituents," *Planta Medica*, vol. 45, pp. 31–34, 1982.Available at: https://doi.org/10.1055/s-2007-971236.

[28] D. J. Finney, *Probit analysis: A statistical treatment of the sigmoid response curve.* Cambridge: Cambridge University Press, 1952.

[29] Z. R. Amelia and A. S. Wulandari, "Effect of 6-BAP application on shoot production of Melaleuca alternifolia seedlings," presented at the in IOP Conference Series: Earth and Environmental Science, 2020.

[30] E.-N. E. M. Mawahib, L. Mohammed, and B. Saeed, "In vitro callus induction of fenugreek (Trigonellafoenum-graecum L.) Using different media with different auxins concentrations," *Agriculture and Biology Journal of North America*, vol. 4, pp. 243-251, 2013.Available at: https://doi.org/10.5251/abjna.2013.4.3.243.251.

© 2022 AESS Publications. All Rights Reserved.

[31] M. E. Elnour, A. Ali, and B. Saeed, "Effect of different concentrations of auxins and combination with kinetin on callus initiation of Trigonella Foenum-Graecum. L," *International Journal of Technical Research and Applications*, vol. 3, pp. 117-122, 2015.

[32] K. G. Abd Elaleem, M. M. Ahmed, and B. E. A. Saeed, "Study of the in vitro callus induction Trigonella foenum-graecum L. from cotyledons and hypocotyls explants supplemented with various plant hormones," *International Journal of Current Microbiology and Applied Science*, vol. 3, pp. 486-493, 2014.

[33] D. A. Bakar, B. A. Ahmed, and R. M. Taha, "In vitro callus induction and plant regeneration of Celosia argentea-an important medicinal plant," *Brazilian Archives of Biology and Technology*, vol. 57, pp. 860-866, 2014.Available at: https://doi.org/10.1590/s1516-89132014026l1.

[34] C. Clarkson, V. J. Maharaj, N. R. Crouch, O. M. Grace, P. Pillay, M. G. Matsabisa, N. Bhagwandin, P. J. Smith, and P. I. Folb, "In vitro antiplasmodial activity of medicinal plants native to or naturalised in South Africa," *Journal of Ethnopharmacology*, vol. 92, pp. 177-191, 2004.Available at: https://doi.org/10.1016/j.jep.2004.02.011.

[35] M. Ouzir, K. El Bairi, and S. Amzazi, "Toxicological properties of fenugreek (Trigonella foenum graecum)," *Food and Chemical Toxicology*, vol. 96, pp. 145-154, 2016.Available at: https://doi.org/10.1016/j.fct.2016.08.003.

[36] M. Hagos and B. S. Chandravanshi, "Levels of essential and toxic metals in fenugreek seeds (Trigonella Foenum-Graecum L.) cultivated in different parts of Ethiopia," *Brazilian Journal of Food Technology*, vol. 19, p. e2015059, 2016.Available at: https://doi.org/10.1590/1981-6723.5915.

[37] A. Rouane, D. Chabane, and K. Arab, "Evaluation of acute toxicity and anti-inflammatory activity of callus extracts of pulicaria incisa (Lam.) Dc," *Applied Ecology And Environmental Research*, vol. 17, pp. 1013-1025, 2019.Available at: https://doi.org/10.15666/aeer/1701_10131025.

Views and opinions expressed in this article are the views and opinions of the author(s), Journal of Asian Scientific Research shall not be responsible or answerable for any loss, damage or liability etc. caused in relation to/arising out of the use of the content.

© 2022 AESS Publications. All Rights Reserved.